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Selection of distinct conformational states of the 5-HT_3 receptor by full and partial agonists

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1 5-Hydroxytryptamine 5-HT_3 receptor-mediated ion currents evoked by 5-HT, quaternary 5-HT (5-HTQ), *meta*-chlorophenylbiguanide (mCPBG), dopamine and tryptamine in N1E-115 mouse neuroblastoma cells have been investigated in whole-cell voltage clamp and single channel patch clamp experiments.

2 The concentration-dependent activation and desensitization of the ion currents evoked by the agonists yield the potency order: mCPBG > 5-HTQ ≈ 5-HT > > tryptamine > dopamine, and the efficacy order: 5-HT ≈ mCPBG ≈ 5-HTQ > > dopamine ≈ tryptamine. Thus, 5-HT, 5-HTQ and mCPBG are full agonists, whereas dopamine and tryptamine are partial agonists at the 5-HT_3 receptor.

3 Full and partial agonists cause complete cross-desensitization and activate single channels with similar conductances and open lifetimes. This shows that full and partial agonists act on the same population of 5-HT_3 receptors.

4 The time course of recovery from desensitization depends on the agonist used. Recovery from partial agonist-induced desensitization is single exponential, whereas the desensitization induced by full agonists recovers with sigmoid kinetics, suggesting at least 3 steps between 4 states.

5 During the process of recovery from cross-desensitization, the full agonists activate a larger fraction of the 5-HT_3 receptors than the partial agonists, irrespective of the agonist used to induce desensitization.

6 It is concluded that full and partial agonists induce distinct desensitized states and, during recovery from desensitization, recognize distinct conformations of unoccupied 5-HT_3 receptors. This conformational selection is likely to account for the different efficacies of full and partial 5-HT_3, receptor agonists.

Keywords: 5-HT_3 receptor; ligand-gated ion channel; single channel patch clamp; whole-cell voltage clamp; desensitization; agonist efficacy; conformational selection; N1E-115 neuroblastoma cells

Introduction

The 5-HT_3 receptor is a ligand-gated ion channel with properties similar to nicotinic acetylcholine (nACh) receptors. These properties include molecular structure (Maricq *et al.*, 1991) and agonist-induced activation and desensitization of ion current (Neijt *et al.*, 1989; Eiselé *et al.*, 1993; Yakel *et al.*, 1993), which is mediated by cation channels (Yakel *et al.*, 1990; Yang, 1990). In mouse N1E-115 neuroblastoma cells, 5-HT_3 receptors have been investigated in detail. From these cells the functional characteristics of the 5-HT_3 receptor-mediated macroscopic whole-cell ion current (Neijt *et al.*, 1986; 1988; 1989; Lambert *et al.*, 1989) and microscopic single channel events (van Hooft *et al.*, 1994; van Hooft & Vijverberg, 1995), as well as the ligand-binding profile of the recognition site (Hoyer & Neijt, 1988; Lummis *et al.*, 1990) and the primary sequence of the cloned 5-HT_3 receptor subunit (5-HT_3R-A) and a splice variant (5-HT_3R-A_s) have been reported (Hope *et al.*, 1993).

Application of the agonist 5-HT to whole-cell voltage clamped N1E-115 cells induces a rapidly activating cation current, which desensitizes in the continuous presence of the agonist (Neijt *et al.*, 1989). Similar transient ion currents are evoked by the application of other 5-HT_3 receptor agonists, e.g., dopamine (Neijt *et al.*, 1986), 2-methyl-5-HT and *meta*-chlorophenylbiguanide (mCPBG) (Sepúlveda *et al.*, 1991). Dopamine acts as a low-affinity, partial agonist on the 5-HT_3 receptor, with an efficacy of approximately 40% of that of 5-HT, as measured by the amplitude ratio of the maximum inward currents induced by the two agonists (Neijt *et al.*, 1986). The selective 5-HT_3 receptor agonist, 2-methyl-5-HT, which binds to 5-HT_3 receptors in N1E-115 cells with similar affinity to 5-HT (Hoyer & Neijt, 1988), is also a partial agonist with an efficacy of 63% on 5-HT_3R-A and of only 9% on 5-HT_3R-A_s homopentameric receptors expressed in *Xenopus* oocytes (Downie *et al.*, 1994). The agonists mCPBG and 5-HTQ are high-affinity receptor ligands and the former has been shown to act as a full 5-HT_3 receptor agonist in N1E-115 cells. The apparent affinities of mCPBG and 5-HTQ obtained from radioligand binding experiments are much higher than those obtained from functional 5-HT_3 receptor assays, in which these agonists are nearly equipotent to 5-HT (Richardson *et al.*, 1985; Glennon *et al.*, 1991; Sepúlveda *et al.*, 1991; Boess *et al.*, 1992; Lummis *et al.*, 1993).

The mechanisms that determine the efficacy of agonists at ligand-gated ion channels are still obscure. Two general principles proposed to account for differences in agonist efficacy are conformational induction and conformational selection (Kenakin, 1995). For some ligand-gated ion channels effects of different agonists have been compared at the single channel level. Unitary events induced by a range of nACh receptor agonists appear to have the same single channel conductance (Gardner *et al.*, 1984), but different open and closed channel lifetimes (Colquhoun & Sakmann, 1985; Papke *et al.*, 1988). For $GABA_A$ receptors, it has been reported that different agonists preferentially activate distinct subconductance states of the ligand-gated Cl^- channel (Mistry & Hablitz, 1990). Both mechanisms may reflect differences in agonist efficacy resulting from a mechanism involving conformational induction. In N1E-115 cells, 5-HT_3 receptor-gated single channel

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events with 4 conductance states have been characterized in the presence of 5-HT (van Hooft *et al.*, 1994; van Hooft & Vijverberg, 1995). In order to demonstrate differences in 5-HT$_3$ receptor agonist efficacy, we have compared the concentration-dependent activation and desensitization of ion currents evoked by full and partial agonists in whole-cell voltage clamp experiments. To examine the mechanisms underlying these differences, single channel events induced by full and partial agonists in cell-attached patches of N1E-115 neuroblastoma cells have been compared and additional kinetic experiments have been performed.

Methods

Cell culture

Mouse neuroblastoma cells of the clone N1E-115 (Amano *et al.*, 1972) were grown under conditions identical to those described before by Neijt *et al.* (1989). Differentiation of subcultures of passage numbers 32-42 was initiated on day 2 in subculture by the addition of 1 mM dibutyryl-cyclic-AMP and 1 mM 3-isobutyl-1-methylxanthine to the culture medium. This medium was refreshed each 2–3 days. Cells were used for experiments on day 7–14 after subculture.

Whole-cell voltage clamp experiments

Membrane currents were recorded by a suction pipette technique for whole-cell voltage clamp as described by Lee *et al.* (1978) and Neijt *et al.* (1989). Fire-polished glass pipettes (200–500 kΩ, Clark GC150 borosilicate) were used to obtain tight seal (>30 MΩ) recordings. The cells were continuously perfused with internal solution via a metal capillary inserted close to the tip of the pipette. The capillary also served as current injection electrode. Membrane potential was measured by an Ag/AgCl pellet electrode, connected to the internal solution via a 150 mM KCl/agar bridge (4% w/v). Electrode junction potentials were compensated before each experiment and remained stable within 1 mV. The external solution was clamped at virtual ground. Membrane currents were measured with a current-to-voltage converter incorporated in the virtual ground circuit. Cells were voltage clamped at −70 mV. Series resistance, estimated from the instantaneous voltage jump in response to a constant current stimulus, was compensated for about 70%. The ionic composition of the internal solution was (in mM): K-glutamate 100, Na-HEPES 20 and sucrose 120. The pH was adjusted to 7.25 with L-glutamic acid. The external solution contained (in mM): NaCl 125, KCl 5.5, HEPES 20, $CaCl_2$ 1.8, $MgCl_2$ 0.8, glucose 24 and sucrose 37. The pH was adjusted to 7.3 with approximately 7 mM NaOH.

Agonist application

Cells were continuously superfused with external solution at a minimum flow rate of 1 ml min^{-1} via one of two capillaries (diameter 100 μm) mounted on a stepmotor-operated micromanipulator. Currents were evoked by stepping from the one capillary with external solution to the other capillary with agonist-containing external solution. The latter capillary was connected with the common outlet of a 4-way valve and could be flushed in between stimuli with external solution containing any desired agonist concentration. The rate of solution exchange was measured from the change in holding current caused by switching the superfusion of a whole-cell voltage clamped cell from external to internal solution. The time constant of solution exchange obtained in this way was 200 ms, which is at least one order of magnitude smaller than any of the time constants reported in this study. Unless the experimental protocol required otherwise, cells were exposed to agonists at sufficiently long intervals to allow complete recovery from desensitization.

Single channel patch clamp experiments

5-HT$_3$ receptor-gated single channels were recorded in the cell-attached patch configuration with an EPC-7 patch clamp circuit (List-Electronic, Darmstadt, Germany) as described previously (van Hooft & Vijverberg, 1995). Borosilicate glass pipettes (10–20 MΩ) filled with agonist-containing external solution were positioned against the cell membrane. From each patch, 50 s of agonist-induced single channel activity was recorded immediately after gigaseal formation. The pipette solution contained (in mM): NaCl 180, HEPES 10, $CaCl_2$ 1.8, $MgCl_2$ 0.8. (pH adjusted to 7.3 with approximately 2.5 mM NaOH).

Whole-cell and single channel ion currents were low-pass filtered (8-pole Bessel filter, corner frequency 600 Hz), digitized (12 bits, 1024 points/record) and stored on magnetic disk for off-line analysis. All experiments were performed at room temperature (20–24°C).

Drugs

Aliquots of frozen stock solutions of 5-hydroxytryptamine creatinine sulphate (Sigma, St. Louis, U.S.A.), tryptamine hydrochloride (Aldrich, Bornem, Belgium), dopamine hydrochloride, 3- tropanyl-3,5-dichlorobenzoate (MDL 72222), N,N,N-trimethyl-5-hydroxytryptamine iodide (5-HTQ) and *meta*-chlorophenyl-biguanide hydrochloride (Research Biochemicals Inc., Natick, U.S.A.) were thawed prior to the experiments and diluted in external solution.

Curve fitting

Parameter estimates of concentration-effect curves were obtained by fitting the function:

$$I = I_{max}/\{1 + (EC_{50}/[agonist])^{n_H}\} \quad [1]$$

The sigmoid onset of recovery from desensitization induced by some of the agonists suggests a multistep mechanism. Since the agonist is rapidly removed during washing the simplest irreversible process with an unknown number of steps, each with identical rate constant α was assumed:

$$D \xrightarrow{n\alpha} D \xrightarrow{(n-1)\alpha} D...D \xrightarrow{\alpha} R \quad [2]$$

where D denotes the desensitized receptor states and R denotes the state of the receptor that has recovered from desensitization. For n steps, i.e., n+1 states, the rate of arrival in R as a function of time, given that the system starts in the leftmost state D at t=0, can be derived and integrated to yield:

$$R(t) = 1 - \exp(-t/\tau)^n \quad [3]$$

in which $\tau = 1/\alpha$. Note that for $n = 1$ this is a monoexponential function. All functions were fitted using a Levenberg-Marquardt non-linear least-squares algorithm (Marquardt, 1963).

Single channel data analysis

In cell-attached patches of N1E-115 cells, 5-HT$_3$ receptor-gated single channels have a main conductance level of 27 pS and subconductance levels of 18, 11 and 6 pS. The probability of occurrence of the 27 pS level is defined as the time spent in the 27 pS level expressed as a percentage of the total channel open time (van Hooft & Vijverberg, 1995). In order to determine the channel open time irrespective of conductance level, events were detected using a detection threshold of 15% of the maximum channel amplitude. A minimum event width of 2.5 ms was imposed. With this time resolution the open times

represent burst length of the channel. Open time distributions are presented as frequency density histograms divided into bins of approximately equal frequency with a minimum of 5 events/bin. Exponential functions were fitted to the events observed by minimizing χ^2 (Marquardt, 1963), and evaluated by testing with the χ^2 goodness-of-fit test (Bendat & Piersol, 1971). The areas under the fitted curve left and right of the shortest and longest event observed were calculated in order to obtain estimates of the percentages of short, respectively long events missed. Maximum-likelihood mean open times were calculated according to Horn & Lange (1983).

All results are expressed as mean ± s.d. of n independent experiments and compared by Student's t test.

Results

Potencies and efficacies for 5-HT$_3$ receptor activation

The superfusion of whole-cell voltage clamped N1E-115 cells with 5-HT, 5-HTQ, mCPBG, dopamine and tryptamine evoked transient inward currents (Figure 1a). These responses were completely and reversibly blocked in the presence of 50 nM of the selective 5-HT$_3$ receptor antagonist, MDL 72222, indicating that all agonist-evoked inward currents were mediated by 5-HT$_3$ receptors (not shown). Figure 1a shows that the inward currents, evoked by concentrations of the various agonists which induced a nearly maximum inward current (see below), had different kinetics and amplitudes. The time constants of inward current decay were 4.2 ± 1.0 s (n = 16) in the presence of 10 μM 5-HT, and 5.8 ± 1.3 s (n = 10) and 3.9 ± 1.5 s (n = 5) in the presence of 10 μM 5-HTQ and 10 μM mCPBG respectively. In addition, time constants of decay of 11.2 ± 5.1 s (n = 8) for 1 mM dopamine- and of 22.7 ± 6.0 s (n = 6) for 100 μM tryptamine-induced inward current were obtained. Since it has been shown previously that the rate of decay of the 5-HT-induced inward current, i.e., the rate of onset of desensitization, saturates at high agonist concentrations (Neijt *et al.*, 1989), the different kinetics reflect differences in the intrinsic ability of the various agonists to induce desensitization of the 5-HT$_3$ receptor-mediated ion current.

Figure 1b shows the concentration-effect curves of the five agonists. The data show that 5-HT and 5-HTQ were equipotent 5-HT$_3$ receptor agonists, and that mCPBG was slightly more potent. The agonists 5-HT, 5-HTQ and mCPBG have high efficacies in evoking 5-HT$_3$ receptor-mediated inward current, as the fitted E_{max} values of the concentration-effect curves of 5-HTQ and mCPBG amounted to 80.6 ± 1.9% (n = 4) and 87.3 ± 2.4% (n = 3) of that of 5-HT, respectively. Dopamine and tryptamine were much less potent than the other agonists investigated and also appear to be partial agonists of the 5-HT$_3$ receptor with efficacies of 36.1 ± 7.5% (n = 4) and 33.0 ± 3.8% (n = 4), respectively. Table 1 summarizes the EC_{50} values, Hill coefficients, and E_{max} values obtained from the concentration-effect curves of the various agonists.

Agonist-induced desensitization

Despite the fact that high concentrations of some agonists failed to induce a full-sized 5-HT$_3$ receptor-mediated inward current, continuous superfusion of the agonists caused complete desensitization. The concentration-dependence of steady-state desensitization was investigated by superfusion of whole-cell voltage clamped cells with external solution containing low concentrations of the agonist for a sufficiently long period before evoking a near-maximum inward current by switching to external solution containing a high concentration of the agonist. Figure 2 demonstrates that complete desensitization was induced by high concentrations of all agonists. In addition, 5-HT and 5-HTQ were equipotent, mCPBG was slightly more potent, and dopamine and tryptamine were much less potent in inducing desensitization. For each agonist the IC_{50} value obtained from the steady-state desensitization curve was below the EC_{50} value for activation of inward current and the Hill coefficients of the desensitization curves were higher than those of the activation curves (Table 1). Figure 2 and Table 1 show that the potency orders for activation and desensitization were identical and that full as well as partial agonists were able to desensitize completely the 5-HT$_3$ receptor-mediated inward current. For the partial agonist, dopamine, a similar concentration-dependent, complete desensitization of the 5-HT$_3$ receptor-mediated membrane depolarizing responses in N1E-115 cells has been demonstrated before (Neijt *et al.*, 1988).

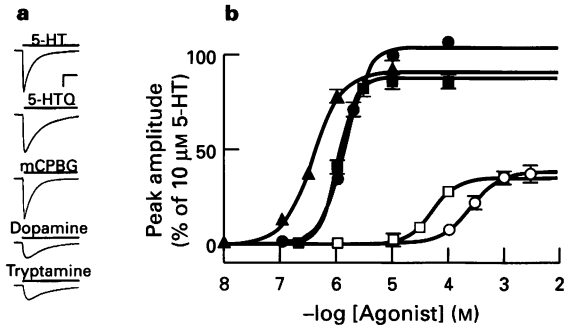

Figure 1 Agonist profile of the 5-HT$_3$ receptor in N1E-115 cells. (a) Whole-cell ion currents evoked by superfusion of voltage clamped cells with 10 μM 5-HT, 10 μM 5-HTQ, 10 μM mCPBG, 1 mM dopamine and 100 μM tryptamine. Each current trace was recorded from a different cell and the amplitudes of the responses have been scaled to that of a 10 μM 5-HT-induced response in the same cell. The bars on top of the records indicate the periods of agonist application. Scale bars: abscissae 10 s, ordinates 2 nA (5-HT, 5-HTQ) and 5 nA (mCPBG, dopamine, tryptamine). (b) Concentration-effect curves of the agonist-induced inward currents (▲ mCPBG; ● 5-HT; ■ 5-HTQ; □ tryptamine; ○ dopamine). All current amplitudes are normalized to the 10 μM 5-HT-induced current. All data points are the means of 3–4 cells. Absence of error bars indicates that the standard deviation is smaller than the symbol size.

Table 1 Potencies, efficacies and Hill coefficients for activation (EC_{50}) and desensitization (IC_{50}) of 5-HT$_3$ receptor-mediated inward current in whole-cell voltage clamped N1E-115 neuroblastoma cells

	EC_{50} (μM)	n_H	E_{max} (%)	n	IC_{50} (μM)	n_H	n
5-HT	1.4 ± 0.2	2.0 ± 0.2	100	3	0.220 ± 0.013	−3.2 ± 0.3	3
5-HTQ	1.1 ± 0.3	2.3 ± 0.5	80.6 ± 1.9	4	0.240 ± 0.014	−2.9 ± 0.4	3
mCPBG	0.40 ± 0.03	1.7 ± 0.2	87.3 ± 2.4	3	0.054 ± 0.002	−2.8 ± 0.2	3
Dopamine	253 ± 27	1.8 ± 0.1	36.1 ± 7.5	4	50 ± 2.0	−2.8 ± 0.3	3
Tryptamine	44 ± 12	2.0 ± 0.2	33.0 ± 3.8	4	17 ± 1.0	−2.7 ± 0.4	3

Values represent mean ± s.d. of parameter estimates obtained from n fitted concentration-effect curves. E_{max} values are normalized to the fitted E_{max} value of 5-HT.

As these results do not exclude the possibility that partial agonists act on a subpopulation of 5-HT$_3$ receptors in N1E-115 cells, cross-desensitization between full and partial agonists was examined. Figure 3 shows that high concentrations of dopamine and tryptamine completely desensitized the 5-HT-induced inward current and that the dopamine- and tryptamine-induced currents were completely desensitized by the prior superfusion of the cells with 5-HT. The complete cross-desensitization demonstrates that the partial and full agonists act on the same population of 5-HT$_3$ receptor-gated ion channels.

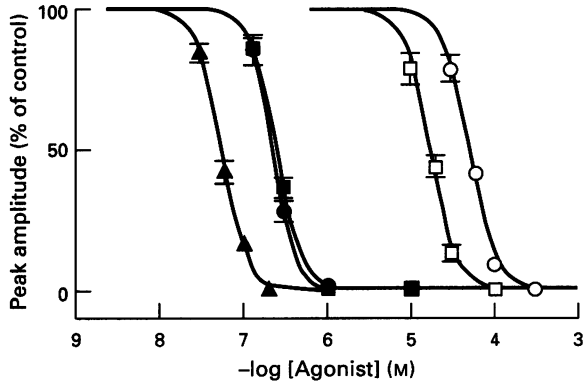

Figure 2 Concentration-effect curves of agonist-induced steady-state desensitization. Whole-cell voltage clamped cells were exposed to low agonist concentrations for 5–12 min before a test response was evoked with a high concentration of the same agonist. For each agonist, responses were normalized to the maximum inward current that could be evoked with that agonist (▲ mCPBG; ● 5-HT; ■ 5-HTQ; □ tryptamine; ○ dopamine). Note that all agonists are able to desensitize the 5-HT$_3$ receptor-mediated inward current completely. All data points are the means of 3 cells. Absence of error bars indicates that the standard deviation is smaller than the symbol size.

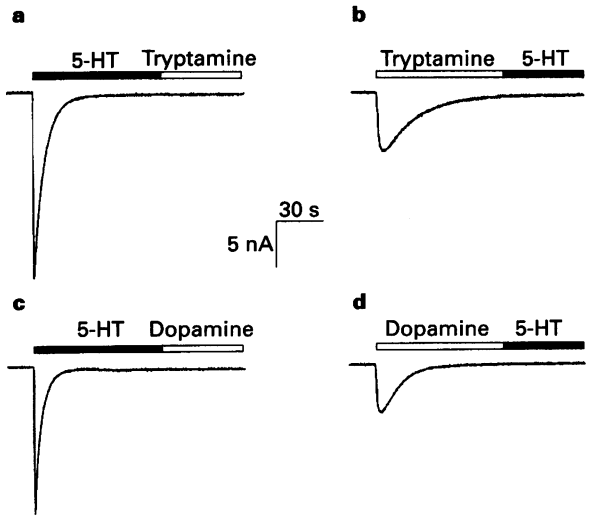

Figure 3 Cross-desensitization between full and partial agonists. (a) A whole-cell inward current was evoked by 10 μM 5-HT (solid bar). After complete desensitization of the ion current, the superfusion was switched to external solution containing 100 μM tryptamine (open bar), which did not result in an inward current. In the reverse protocol, application of 10 μM 5-HT did not evoke an inward current when the receptor was desensitized with 100 μM tryptamine (b). (c,d) Same protocol as in (a) and (b), but with 1 mM dopamine instead of 100 μM tryptamine. Traces are consecutive records obtained from the same cell. Similar results were obtained in two other cells.

Single channel characteristics

In order to investigate whether the 60% difference in efficacy between full and partial agonists related to differences in single channel properties, patch clamp experiments were performed. Single channel events induced in cell-attached patches by EC$_{50}$ concentrations of 250 μM dopamine and of 0.4 μM mCPBG were similar to those induced by an EC$_{50}$ concentration of 1.5 μM 5-HT (Figure 4). The main conductance levels of the 5-HT-, dopamine- and mCPBG-induced events, as obtained from single channel *I-V* curves (Figure 4), were 27.8±1.7 pS (n=4), 27.6±2.1 pS (n=5) and 27.2±2.4 pS (n=3), respectively. In addition, for single channel events evoked by 5-HT, dopamine and mCPBG, the probability of occurrence of the 27 pS level was 68.4±9.9% (n=3), 70.1±11.5% (n=3), and 74.0±5.5% (n=3) of the total open time. These values do not differ significantly (P=0.44–0.86), indicating that the single channel conductance and the probabilities of occurrence of the various conductance levels do not depend on the agonist used. The open time distribution of events induced by the EC$_{50}$ concentration of 1.5 μM 5-HT was fitted by a single exponential function with a time constant of 23.9±2.0 ms (9.8% missed short events, 0.1% missed long events, P=0.62, χ^2 goodness-of-fit). Open time histograms of events induced by EC$_{50}$ concentrations of 250 μM dopamine and of 0.4 μM mCPBG were also fitted by single exponential functions with similar time constants of 23.5±2.4 ms (9.6% missed short events, 0.3% missed long events, P=0.39) and 28.8±2.4 ms

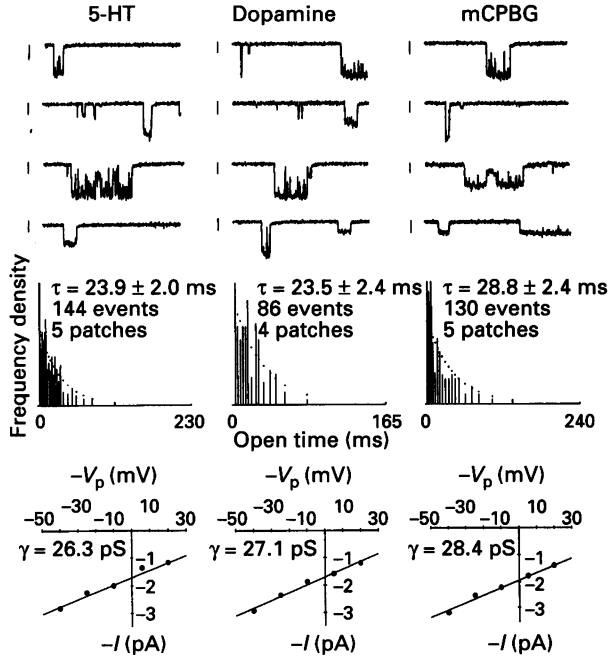

Figure 4 Agonist-evoked single channel events recorded from cell-attached patches at a pipette potential of +40 mV. The full agonists, 5-HT and mCPBG, as well as the partial agonist, dopamine induce multiple conductance levels. Records of 500 ms were selected from different patches and have been scaled to match the amplitude of the 27 pS level. Frequency density histograms of single channel open times were collected from the number of patches indicated and are well fitted by single exponential functions (χ^2 goodness-of-fit test P=0.39–0.62). The fitted exponential time constants and estimated s.d. values, as obtained from the fitting procedure, are indicated in each histogram. Bars represent the observed frequency densities of open times and dots are fitted values. The single channel *I-V* curves in the lower panel were obtained from the amplitude of the main conductance level of single channel events in cell-attached patches at the pipette potentials indicated. The single channel conductances (γ) were obtained from the slopes of the *I-V* curve by linear regression (solid lines). Scale bars left to the single channel records indicate 1 pA.

(8.8% missed short events, 1.1% missed long events, $P=0.43$), respectively. These values compare to the maximum-likelihood mean open time estimates, which were 24.9 ± 5.7 ms, 24.3 ± 9.9 ms and 23.9 ± 5.7 ms for 5-HT, dopamine and mCPBG, respectively. The similar values of the single channel parameters, demonstrate that the large difference in efficacy between the full agonists 5-HT and mCPBG and the partial 5-HT$_3$ receptor agonist, dopamine, cannot be accounted for on the basis of channel conductance or burst length, suggesting that the partial agonist is less efficacious in activating otherwise similar ion channels.

Dynamic equilibria between receptor states

The similarity in open channel parameters suggests that the large differences in efficacy between full and partial 5-HT$_3$ receptor agonists might be due to differences in dynamic equilibria between receptor states, e.g., resting and desensitized states. As the kinetics of most of the various steps of receptor activation and desensitization are either rapid or complex to study, we chose to investigate the recovery from desensitization. This is a relatively simple situation in which complete desensitization is induced and, subsequently, the agonist is washed off rapidly. The fraction of the 5-HT$_3$ receptors available for activation can be measured from the amplitude of the inward current evoked by superfusion of the cell with a near maximum-effective concentration of agonist. By varying the period of washing the kinetics of recovery from desensitization can be obtained (Neijt *et al.*, 1989). Figure 5a shows the time course of recovery from desensitization of the 1 mM dopamine- and 100 μM tryptamine-induced inward currents. Both curves are best fitted by Eqn. [3] with similar values of the exponent of 0.6 ± 0.3 and 0.7 ± 0.2 ($n=3$), but with different time constants. For dopamine the average time constant were 11.2 ± 2.0 s ($n=3$) and for tryptamine 32.2 ± 14.4 s ($n=3$). Recovery from desensitization induced by the full agonists (Figure 5b) appeared sigmoid at early time. Best fits were obtained by Eqn. [3] with exponent values of 2.4 ± 0.3 (5-HT, $n=3$), 2.5 ± 0.2 (5-HTQ, $n=3$) and 2.6 ± 0.5 (mCPBG, $n=3$), and time constants of 11.2 ± 1.3 s ($n=3$) for 10 μM 5-HT-, 15.7 ± 4.6 s ($n=3$) for 10 μM 5-HTQ- and 101 ± 14.1 s ($n=3$) for 10 μM mCPBG-induced desensitization. With mCPBG, approximately 9 min of washing were required for complete recovery from desensitization. When desensitization was induced with 200 nM mCPBG, a concentration just sufficient to induce complete desensitization (see Figure 2), recovery from desensitization showed the same sigmoid time course with a time constant of 107 ± 12.7 s and an exponent of 2.3 ± 0.2 ($n=3$). This confirms our previous finding with 5-HT (Neijt *et al.*, 1989) that the time course of recovery from desensitization is independent of the concentration of agonist used to induce desensitization. In order to exclude the possibility that, during the recovery from desensitization, reassociation of the agonist contributes to the sigmoid time course, 10 mM of the rapidly acting competitive antagonist TEA (Kooyman *et al.*, 1993b) was applied during washing off the agonist. The time constant and exponent of recovery from 5-HT-induced desensitization in the presence of 10 mM TEA were 9.4 ± 1.4 s and 2.8 ± 0.2 ($n=3$). These values are similar to the values obtained without TEA indicating that reassociation of the agonist does not contribute to the sigmoid time course of recovery from desensitization. As outlined in scheme [2], the results suggest that recovery from partial agonist-induced desensitization required at least 1 step between 2 states, whereas recovery from full agonist-induced desensitization required at least 3 steps between 4 states.

Thus far, the recovery from desensitization was monitored with the same agonist as used to induce desensitization. In order to investigate whether recovery from desensitization also depends on the agonist used to monitor the fraction of receptors that has returned to the resting state, recovery from cross-desensitization was examined. For these experiments complete desensitization was induced by one agonist and the recovery was measured with another agonist (Figure 6a). Recovery from tryptamine-induced desensitization after a fixed recovery period of 10 s was monitored using 5-HT, mCPBG, dopamine and tryptamine (Figure 6b). Despite the fact that the agonists were applied at exactly the same stage of recovery from tryptamine-induced desensitization, the apparent recovery of the full agonist-evoked responses was greater than that of the partial agonist-induced responses. This implies that the full agonists activate a larger fraction of the 5-HT$_3$ receptors which have returned to the resting state after 10 s of recovery from tryptamine-induced desensitization. In the reverse experiment the partial agonists activate a smaller fraction of resting 5-HT$_3$ receptors following 20 s recovery from 5-HT-induced desensitization (Figure 6c).

The complete time course of recovery from cross-desensitization was measured for combinations of the full agonist, 5-HT and the partial agonist, tryptamine. The time course of recovery of the 10 μM 5-HT response after desensitization in-

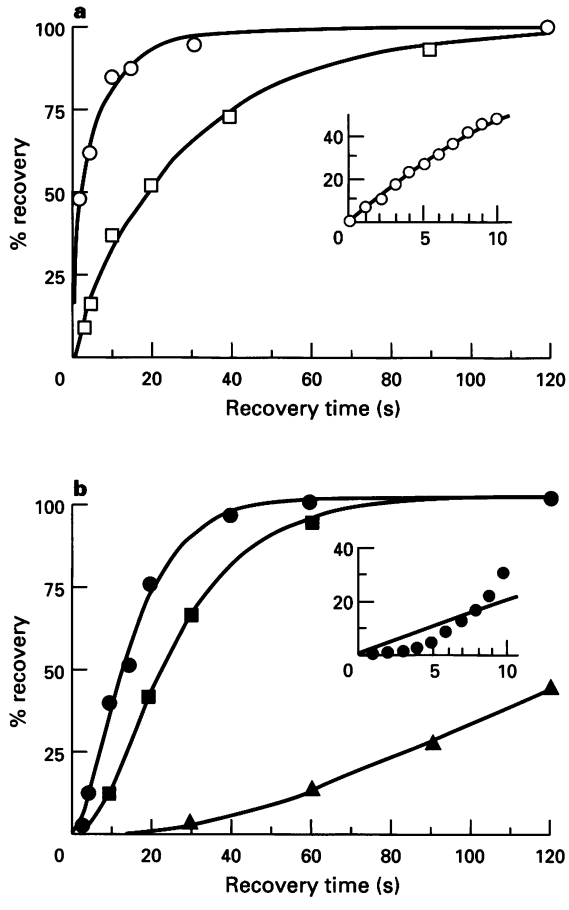

Figure 5 Recovery from agonist-induced desensitization. (a) Desensitization was induced by superfusion of the partial agonists dopamine (1 mM; ○) or tryptamine (100 μM; □) for 2 min. Recovery from desensitization was measured from the amplitude of a test response evoked after a variable period of washing off the agonist. The solid curves are fitted according to Eqn. [3]. The fitted time constants for dopamine and tryptamine are 11.2 s and 30.5 s with exponents of 0.6 and 1.0, respectively. (b) Recovery from desensitization with the full agonists 5-HT (10 μM; ●), 5-HTQ (10 μM; ■) and mCPBG (10 μM; ▲) measured by the same protocol as in (a). Fitted time constants (Eqn. [3]) are 10.5 s, 15.6 s and 86.9 s with exponents of 2.4, 2.8 and 2.9 for 5-HT, 5-HTQ and mCPBG, respectively. For illustrative purposes, the recovery of mCPBG is shown up to 120 s only. Complete recovery of mCPBG-induced desensitization was obtained after 9 min of washing. Insets show two examples of the early time course of recovery from desensitization of tryptamine (inset a) and 5-HT (inset b). The solid curves represent a monoexponential function fitted to the data in order to clearly show the different recovery kinetics from full and partial agonist-induced desensitization.

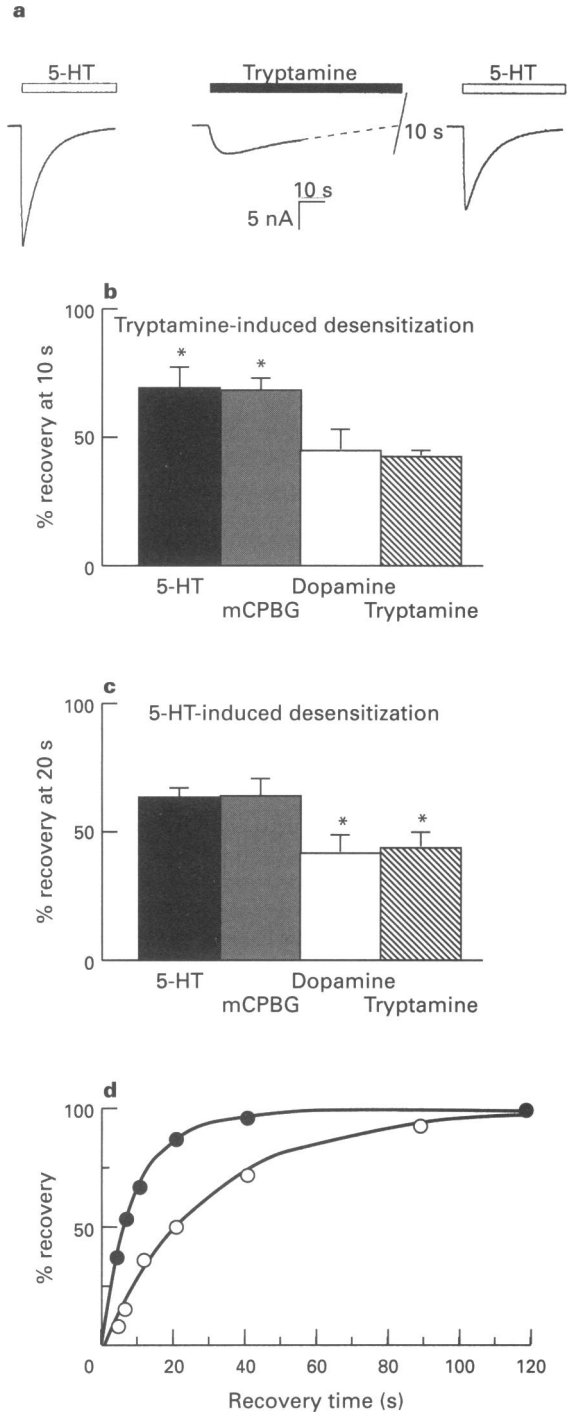

Figure 6 Recovery from cross-desensitization. (a) Experimental protocol with tryptamine as the desensitizing agonist. A control response was evoked with 10 μM 5-HT, the receptor was desensitized with 100 μM tryptamine and after 10 s of washing recovery was monitored with 10 μM 5-HT. The recovery of the 5-HT-induced response after tryptamine-induced desensitization was expressed as percentage of the control 5-HT response. (b) Using the protocol as in (a), the recovery monitored with 10 μM 5-HT (69.1 $\pm$ 8.5%) and 10 μM mCPBG (69.1 $\pm$ 4.6%) is significantly larger than the recovery monitored with 1 mM dopamine and 100 μM tryptamine (45.1 $\pm$ 12.7% and 43.1 $\pm$ 1.9%, respectively). (c) Similar experiments, in which recovery from 5-HT-induced desensitization was measured after 20 s of washing, demonstrate that the recovery monitored with dopamine and tryptamine (42.0 $\pm$ 6.9% and 44.0 $\pm$ 6.1%, respectively) is significantly less than that monitored with 5-HT (62.8 $\pm$ 4.2%) and mCPBG (63.9 $\pm$ 7.0%). Columns in (b) and (c) are mean $\pm$ s.d. of data obtained from three cells. Significant differences ($P<0.05$) compared with tryptamine (b) and with 5-HT (c) are indicated by asterisks. (d) Time course of recovery from tryptamine-induced desensitization obtained by using the protocol as in (a) with a variable period of washing. The curve fitted according to Eqn. [3] to the results obtained yields a time constant and an exponent of 12.4 s and 0.8 for the recovery monitored with 5-HT (●), as compared to 30.5 s and 1.0 for the recovery monitored with tryptamine (○, data from Figure 5a).

duced with 100 μM tryptamine (Figure 6d) was fitted by Eqn. [3] with a time constant of 13.7 $\pm$ 2.0 s and an exponent of 0.7 $\pm$ 0.2 ($n=3$). Similar experiments were performed in which desensitization was induced with 10 μM 5-HT and recovery was measured with 100 μM tryptamine (not shown). These experiments yielded a time constant of recovery of 13.8 $\pm$ 0.7 s and an exponent of 3.1 $\pm$ 0.4 ($n=3$). Comparison of the curves fitted to the recovery from desensitization (Figure 5) with those fitted to the recovery from cross-desensitization shows that the values of the exponent, i.e. the number of steps between the desensitized and resting states, depends on the agonist used to induce desensitization.

Discussion

The efficacies of 5-HTQ and mCPBG in inducing 5-HT$_3$-receptor-mediated ion current in mouse N1E-115 cells are nearly as high as that of 5-HT (Table 1), indicating that these compounds act as full agonists on 5-HT$_3$ receptors. Conversely, dopamine and tryptamine are partial agonists with efficacies of approximately 40% of that of the full agonists. The potencies of the full agonists are in the low micromolar range, whereas the two partial agonists are more than two orders of magnitude less potent. For all agonists, the IC_{50} values obtained from steady-state desensitization curves are smaller than the corresponding EC_{50} values obtained from the peak amplitude of transient inward currents by a factor of 3–7. The desensitization curves are steeper than the activation curves as reflected by the differences in Hill coefficients (Table 1). Although this indicates a difference in cooperativity between the activation and desensitization mechanisms, it should be noted that steady-state conditions cannot be assumed at the time of peak amplitude inward current.

Apparent K_D values of 380–560 nM of 5-HT to displace 5-HT$_3$ receptor antagonists in N1E-115 cells (Hoyer & Neijt, 1988; Lummis *et al.*, 1990) and the IC_{50} value of 220 nM of the desensitization curve of 5-HT are within a narrow range. Similarly, the IC_{50} of 35.5 μM of tryptamine to displace [^{3}H]-zacopride bound to 5-HT$_3$ receptors in rat cortical membranes (Alhaider *et al.*, 1993) is close to the IC_{50} value of 17 μM obtained from the desensitization curve of tryptamine (Table 1). The comparison shows that the affinities of 5-HT and tryptamine for the recognition site are equal to or slightly below their potencies in inducing desensitizations. Conversely, apparent binding affinities of 5-HTQ ($K_i=17$ nM; Glennon *et al.*, 1991) and mCPBG ($IC_{50}=2$ nM; Sepúlveda *et al.*, 1991) are much higher than their potencies for inducing desensitization. Binding experiments with radiolabelled mCPBG have shown two populations of high-affinity sites in N1E-115 cells ($K_{D1}=0.03$ nM, $K_{D2}=4.4$ nM; Lummis *et al.*, 1993) and it has been suggested that the high binding affinity of this agonist is related to an enhanced affinity of the 5-HT$_3$ receptor in the desensitized state. The present results do not confirm these findings, as the desensitization curve of mCPBG reveals only one population of sites, which are much less sensitive to the agonist as compared to the binding affinities reported. Results on other high-affinity 5-HT$_3$ receptor agonists from both functional and radioligand-binding assays are required to resolve the discrepancy between radioligand binding affinities and functional potencies at the 5-HT$_3$ receptor.

From molecular modelling with structurally distinct 5-HT$_3$ receptor antagonists, ligand interaction with the 5-HT$_3$ receptor has been proposed to involve a pharmacophore with a cationic and an aromatic binding site (Gozlan & Langlois,

1992). Agonist effects also seem to involve aromatic and cationic interactions with the 5-HT$_3$ receptor as demonstrated by the distinct effects of the quarternary ammonium ion TEA, which resembles the cationic moiety of the 5-HTQ molecule and competitively blocks 5-HT$_3$ receptor-mediated inward current (Kooyman *et al.*, 1993b), and 5-hydroxyindole, the aromatic moiety of 5-HT, which prevents desensitization of the inward current (Kooyman *et al.*, 1993a; 1994). The present results show that the quaternary agonist, 5-HTQ and 5-HT have identical potencies and efficacies, indicating that the small change of the cationic moiety of 5-HT has little effect on agonist interaction. Conversely, the potency and efficacy of tryptamine, which is identical to 5-HT except for the absence of the hydroxyl residue in the aromatic moiety, are greatly reduced as compared to 5-HT. This indicates that changes of the aromatic moiety of the agonist greatly affect 5-HT$_3$ receptor agonist potency and efficacy.

The large differences in efficacy between the full agonists 5-HT, 5-HTQ and mCPBG and the partial agonists dopamine and tryptamine cannot be accounted for by various plausible mechanisms: (1) Open time distribution and conductance of single channel events induced by the partial agonist dopamine are very similar to those induced by the full agonists 5-HT and mCPBG (Figure 4). With a more detailed analysis of the single channel kinetics, as performed previously for nACh receptor-gated ion channels (Colquhoun & Sakmann, 1985), subtle differences in full and partial agonist-induced single channel kinetics might be revealed. At present however, such an analysis of 5-HT$_3$ receptor-mediated single channels is hampered by the low event frequency (Figure 4) and desensitization of the channels in the cell-attached patch configuration. Nonetheless, within the limits of the present experimental accuracy, the 60% difference in efficacy of 5-HT$_3$ receptor agonists cannot be explained by differences in channel conductance and/or burst length. The only single channel parameter that relates to the different effects of the agonists is the probability of channel opening (P_O), as reflected by the different event frequencies observed (Figure 4). (2) Although the partial agonist-induced currents activate more slowly than the full agonist-induced currents, activation kinetics are always much more rapid than desensitization kinetics (Figure 1a). Any underestimation of the peak amplitudes of the more rapid, full agonist-induced currents would result in a yet greater difference in efficacy between full and partial agonists. (3) The possibility that partial agonists act on a subset of 5-HT$_3$ receptors can also be excluded, since the partial agonists cause complete desensitization of the full agonist-induced currents (Figure 3). In this respect the expression of both 5-HT$_3$R-A and 5-HT$_3$R-A$_s$ mRNA's in N1E-115 cells (Hope *et al.*, 1993) is of particular interest. Previously we have found that, in the differentiated N1E-115 cells, the efficacy of 2-methyl-5-HT is approximately 75% of that of 5-HT (Kooyman *et al.*, 1993a), suggesting the predominant expression of the 5-HT$_3$R-A subunit (Downie *et al.*, 1994). Moreover, all concentration-effect curves presented (Figures 1b and 2) are monophasic and do not suggest the existence of receptor populations with distinct affinities.

The present results show that full and partial agonist-induced inward currents have different kinetics with respect to both activation and desensitization of the inward current and recovery from desensitization. Recovery from desensitization induced by 5-HT is not monoexponential (Figure 5b). In a previous study (Neijt *et al.*, 1989), we have corrected a delay in the recovery from 5-HT-induced desensitization, which was attributed to the delay in the superfusion system. However, the comparison of rapid (dopamine) and slow (mCPBG) recovery curves presented in Figure 5 clearly shows that the apparent delay observed with some agonists is not an artefact of the superfusion system. In the present experiments the rate of solution exchange has always been much faster than the rate of recovery from desensitization. Further, as previously shown for 5-HT (Neijt *et al.*, 1989) and confirmed with mCPBG in the present experiments, the rate of recovery from desensitization depends only on the agonist used and not on its concentration. Together with the absence of an effect of the competitive antagonist TEA on the kinetics of recovery from desensitization this indicates that agonist reassociation cannot account for the sigmoid time course. The data on sigmoid recovery from full agonist-induced desensitization (Figure 5b) exclude the possibility that the kinetics are determined by a single rate limiting dissociation step or conformational transition. Therefore, it has been assumed that recovery from desensitization involves multiple steps, which occur at similar rates (scheme [2]). Dissociation of agonist molecules from multiple sites, multiple conformational transitions, or a combination of the two can all be described by the general sequential mechanism as presented in scheme [2]. The fitting of the data by Eqn. [3] with values of the exponent between 0.6 and 0.7 indicates that a minimum of one step is required for the recovery from partial agonist-induced desensitization, whereas recovery from full agonist-induced desensitization requires at least three steps, as indicated by exponential values of 2.4–2.6. The moderate potencies of the agonists suggest that the rate of dissociation is high as compared to the rate of recovery from desensitization (e.g., see Hammes & Schimmel, 1970). The results of Figure 6d are consistent with the notion that agonist dissociation is not the rate limiting step in the recovery from desensitization, since the rate of recovery from tryptamine-induced desensitization when monitored with 5-HT is faster than when monitored with tryptamine. This indicates that some of the steps leading to the resting state are transitions between desensitized states of unoccupied 5-HT$_3$ receptors. The experiments on recovery from cross-desensitization (Figure 6d) confirm that the number of steps from the desensitized to the resting state depends on the agonist used to induce desensitization. Therefore, it is concluded that the partial agonists convert the receptor in a desensitized state that differs from the desensitized state induced by the full agonists. A reported delay in the recovery from desensitization of the ACh-induced ion current in BC3H1 cells (Dilger & Liu, 1992) indicates that a multistep mechanism might also apply to the recovery from desensitization of nACh receptors.

The results of Figure 6 demonstrate that, during the process of recovery from desensitization, the full agonists activate a larger fraction of the 5-HT$_3$ receptors than the partial agonists. Since the concentrations of agonists used in the recovery experiments were sufficiently high to evoke maximum amplitude inward currents and because it has been excluded before that full and partial agonists act on specific subpopulations of 5-HT$_3$ receptors (Figure 3), the results suggest that full and partial agonists recognize distinct conformations of unoccupied, activatable 5-HT$_3$ receptors. The principle of recognition of distinct, coexisting receptor conformations by different agonists has been referred to before as conformational selection (Kenakin, 1995). Since the partial agonists recognize a subset of activatable receptors during recovery from desensitization, it is likely that a similar selection of resting, activatable 5-HT$_3$ receptor conformations is responsible for the large difference in efficacy between full and partial agonists. The slow onset of inward curents induced by high concentrations of partial agonists (Figures 1a and 3) and the lower frequency of the dopamine-induced single channel events are consistent with the notion that the partial agonists act on resting receptor conformations that are less prevalent than those available for the full agonists. As the single channel parameters of full- and partial agonist-induced events are very similar (Figure 4), the data provide no evidence for a role of conformational induction in agonist efficacy.

In conclusion, the results presented in this study provide evidence for a mechanism accounting for 5-HT$_3$ receptor agonist efficacy in terms of conformational selection. Together with the hypothesis on the allosteric behaviour of ligand-gated ion channels (Jackson, 1995; Changeux & Edelstein, 1995), this may provide a framework for understanding the dynamics of agonist-receptor interactions.

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